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Cytochrome *c* oxidase levels in chondrocytes during monolayer expansion and after return to three dimensional culture

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Summary

Objective: Here we investigate whether monolayer culture or culture at 21% oxygen influences activity of cytochrome *c* oxidase, the terminal enzyme in the respiratory chain whose activity is essential for oxidative metabolism and whether return to three dimensional (3-D) culture restores cytochrome *c* oxidase activity to original levels.

Methods: Primary bovine articular chondrocytes were cultured in alginate beads (3-D) for 4 weeks or in monolayer under 1% and 21% oxygen for up to 9 days and then returned to 3-D culture for up to 4 weeks. Cells were stained to localise cytochrome *c* oxidase within the cells. Mitochondrial protein content and cytochrome *c* oxidase enzymatic activity were determined. Expression of cytochrome *c* oxidase subunits, *COXI* and *COXIV*, was assessed by qRT-PCR.

Results: Cytochrome *c* oxidase staining remained minimal in chondrocytes cultured in alginate for 4 weeks under 21% oxygen. Mitochondrial protein content and cytochrome *c* oxidase activity increased significantly during 9 days of chondrocyte expansion in monolayer, accompanied by up-regulation of the *COXI* mitochondrial gene but not the *COXIV* nuclear-encoded gene. Cytochrome *c* oxidase staining increased from day 5 of monolayer culture and remained high even after the cells were returned to 3-D culture for 4 weeks.

Conclusions: Culture of chondrocytes in monolayer leads to a rapid increase in mitochondrial protein content and cytochrome *c* oxidase activity. The increase in cytochrome *c* oxidase activity is not reversed even after chondrocytes are returned to 3-D culture for 4 weeks; high oxygen tension alone does not appear to stimulate cytochrome *c* oxidase activity.

Key words: Cartilage, Mitochondria, Dedifferentiation, Oxygen tension.

Introduction

Autologous chondrocyte transplantation (ACT) has become widely used for treatment of cartilage defects arising from knee injuries[1] and generally requires expansion of cell numbers before treatment. Chondrocytes undergo numerous changes during expansion in monolayer and after returning to a three dimensional (3-D) environment. Primary chondrocytes lose their rounded morphology in monolayer and dedifferentiate into fibroblast-like cells switching their matrix protein synthesis from collagen type II and aggrecan to collagen type I and versican[2–5]. On return to a 3-D environment, the rounded shape and protein synthesis typical of chondrocytes is restored[6–8] though not necessarily completely[9]. It remains an open question as to whether chondrocytes can re-differentiate completely to their original phenotype.

During expansion in monolayer, the environmental conditions of the cells differ from those encountered *in vivo* in the tissue in many respects and in particular in regard to oxygen tension. Chondrocytes are almost invariably expanded under atmospheric conditions (21% O_2) whereas in the joint, oxygen tension is low (~5% O_2)[10] because cartilage is avascular. Chondrocytes in the matrix obtain their energy almost entirely by glycolysis[11–13], a pathway which does not utilise oxygen, and they can survive many days under anoxia[14]. Nevertheless, chondrocytes consume oxygen albeit at low rates compared to other cell types[10,12,15,16] and require oxygen to maintain matrix synthesis and other cellular functions[11,14,17,18]. The pathway of oxidative phosphorylation appears to have been almost completely abandoned by chondrocytes *in situ*. Articular chondrocytes contain only a low number of mitochondria[19,20]. The activity of the enzymes making up the respiratory chain involved in oxidative phosphorylation (complexes I, II, III and IV), which are located on inner mitochondrial membrane, has been found to be low or absent[21]; only cytochrome *b*, a subunit of complex III, has been detected by spectroscopy in freshly isolated chondrocytes[22]. The role of oxygen in cartilage metabolism is not yet understood.

When chondrocytes are cultured in monolayer at 21% oxygen, metabolic pathways appear to alter. Mitochondrial activity and the ratio of mitochondrial DNA to total DNA measured by rhodamine 123 uptake increase within 4 days of monolayer culture with spectroscopic analysis demonstrating the presence of cytochromes in quantities typical for those in a functional respiratory chain[22,23]. All complexes of the respiratory chain appear active in chondrocytes at confluence[24]. Hence the reported *c*.10-fold

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increase in oxygen consumption by chondrocytes cultured in monolayer[25,26] suggests a move to oxidative metabolism.

Here we investigated two questions; first, whether monolayer culture or an increase in oxygen tension *in vitro*[27] affects activity of cytochrome *c* oxidase, the final enzyme of the electron transport chain; second, whether cytochrome *c* oxidase activity of chondrocytes cultured in monolayer decreases when they are returned to 3-D culture.

Materials and methods

MATERIALS

PicoGreen (Molecular Probes Inc), MitoTracker Red CM-H_2XRos (Molecular Probes Inc) and agarose were from Invitrogen (Paisley, UK). The OneStep RT-PCR kit and RNeasy Mini kit were from Qiagen (Crawley, UK). DNase I, DNase I buffer and DNase inactivation reagent were from Ambion (Huntington, UK). TaqMan Universal PCR mastermix, 18S TaqMan gene expression assay and TaqMan gene expression assays-on-demand were from Applied Biosystems (Warrington, UK). Dulbecco's modified Eagles medium (DMEM) and foetal bovine serum (FBS) were from Gibco (Paisley, UK); trypsin-ethylenediaminetetraacetic acid (Trypsin–EDTA) solution, antibiotic/antimycotic mixture (A5955: 10,000 units ml^{-1} penicillin G, 10 mg ml^{-1} streptomycin, 25 μg ml^{-1} amphotericin), collagenase type I and other chemicals were from Sigma–Aldrich (Poole, Dorset, UK); alginate was from Fluka (c/o Sigma–Aldrich Chemie GmbH, Buchs, Switzerland).

METHODS

Cell culture

Chondrocytes were isolated by collagenase digestion[28] from the articular cartilage of metacarpal–phalangeal joints of 18–24 month cattle. Each experiment was performed using cells isolated from a single animal. For each experiment, chondrocytes were cultured continuously in 3-D alginate beads[29] at 4×10^6 cells ml^{-1} for up to 6 weeks or were seeded in monolayer on cover slips at 10^4 cells cm^{-2} and harvested after 3–9 days in culture with 0.25% trypsin–EDTA. They were then either assayed or encapsulated in alginate beads to provide 3-D support and cultured for a further 1–4 weeks. Chondrocytes were released from alginate bead by citrate buffer[28]. Fibroblasts were isolated from skin dermis of the same animals[28] and cultured in monolayer as a positive control for cytochrome *c* oxidase staining. DMEM with 10% FBS, antibiotic/antimycotic mixture ($1 ml \times 100^{-1}$ ml DMEM) and osmolarity adjusted to 380 mOsm with NaCl[28] were used in all experiments. DMEM was changed every 2–3 days to maintain pH levels above pH 7.1 and glucose concentrations above 2 mM. Cells were counted using a haemocytomer and trypan blue at each sampling point. For chondrocytes in monolayer, the total number of population doublings during the exponential growth phase over a 24 h time period was defined as population doubling level (PDL) and calculated according to the formula: $PDL = \log(N_2/N_1)/\log 2$, where N_1 is the initial number of cells and N_2 is the final number of cells released after culture[30].

Cell culture under 1% oxygen. Cell cultures were cultured in sealed modular incubator chambers maintained at 37°C (Billups-Rothenberg Inc, Del Mar, USA) flashed by a gas mixture (1% oxygen, 5% carbon dioxide, 94% nitrogen) for 20 min at the beginning of the incubation and at each medium change. Control cell cultures were kept at 37°C under an air/5% CO_2 mix.

Quantitative measurement of enzyme activity

For each experiment, cells cultured in monolayer were harvested for quantitative enzyme assays at only one time point (at either day 0, 3, 6 or 9; one animal = one observation).

Isolation of mitochondria. Harvested chondrocytes were centrifuged in phosphate buffered saline (PBS) at 750 g for 5 min. The resulting cell pellet was re-suspended in ice-cold buffer containing 0.25 M sucrose, 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 3 mM EDTA, pH 7.2 and homogenised by 10 passes in a glass/glass homogeniser (0.152–0.254 mm pestle clearance) kept on ice. Mitochondria were isolated[31] and the resulting mitochondrial pellet was re-suspended in 60–150 μl buffer (20 mM MOPS, 3 mM EDTA, 0.25 M sucrose, pH 7.2) for measurement of cytochrome *c* oxidase and citrate synthase (CS) enzymatic activities. The protein content of the mitochondrial suspension was measured using a Bio-Rad *DC* protein assay (Bio-Rad, Hemel Hempsted, UK). Mitochondrial protein was solubilised by 0.1% triton X-100 and calibrated against bovine serum albumin containing 0.1% triton X-100. Absorbance at 620 nm was measured on plate reader (Anthos *ht*2, Anthos Labtec Insruments, Austria). The degree of mitochondrial integrity was calculated as a percentage of mitochondria with undamaged membranes: $\% = (U_{total} - U_{damaged}) \times 100 \times U_{total}^{-1}$, where U_{total} was cytochrome *c* oxidase activity of mitochondria whose membranes were disintegrated by *n*-dodecyl β-D-maltoside and $U_{damaged}$ was cytochrome *c* oxidase activity of mitochondria with intact membranes.

Measurement of cytochrome c *oxidase-specific activity*. Cytochrome *c* oxidase (EC 1.9.3.1) activity was assayed from the decrease in absorbance at 550 nm caused by oxidation of ferrocytochrome *c* (reduced form) to ferricytochrome *c* (oxidised form) by cytochrome *c* oxidase[32]. The reaction mix contained an aliquot of mitochondrial suspension, 0.1 M 2-(*N*-morpholino)-ethanesulfonic acid (MES), 0.3 mM *n*-dodecyl β-D-maltoside, 1 mM EDTA, pH 6.0, and cytochrome *c* reduced by ascorbic acid. Reduction of cytochrome *c* (C2506, Sigma–Aldrich, Pool, UK) was performed by adding ascorbic acid to the cytochrome *c* solution (26 mg ml^{-1}) until the colour of the solution became salmon-pink. The excess ascorbic acid was removed by running the reduced cytochrome *c* solution through a PC-10 desalting column (Amersham, Little Chalfont, UK). The ratio A_{550}/A_{565} of a 10-fold diluted aliquot was >10 which characterises sufficient reduction. The column effluent containing reduced cytochrome *c* was added to the reaction mix to give an OD 550 nm ~0.3 (10–20 μl effluent ml^{-1} of final volume of reaction mix). The reaction was initiated by adding the mitochondrial suspension to the reaction mix and changes in absorbance were recorded for 2 min[33]. The difference in extinction coefficients between reduced and oxidised cytochrome *c* was taken as 21.86 mM^{-1} cm^{-1} at 550 nm[34]. The specific cytochrome *c* oxidase activity was expressed in units per mg of mitochondrial protein or per million cells (1 unit of cytochrome *c* oxidase oxidises 1 μmol of ferrocytochrome *c* per min).

Measurement of CS specific activity. CS (EC 4.1.3.7) activity, used as common quantitative marker enzyme for the content of mitochondria in the cells, was assayed by its ability to catalyze the regeneration of acetyl coenzyme A (acetyl CoA) in the presence of oxaloacetate to CoA[35]. CoA in turn reacted with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form the coloured product thionitrobenzoic acid (TNB) (recorded at 412 nm at room temperature); 1 ml of reaction mix contained 10–20 μl aliquot of mitochondrial suspension (*c*.1 mg ml^{-1} mitochondrial protein), 0.07 mg ml^{-1} oxaloacetate, 0.25 mg ml^{-1} acetyl coenzyme A lithium salt, 0.04 mg·ml^{-1} DTNB, and 0.1% triton X-100 in 100 mM Tris buffer of pH 8.0. The extinction coefficient[35] of TNB was taken as 13.6 mM^{-1} cm^{-1}. CS specific activity was expressed in units per mg of mitochondrial protein or per million cells; 1 unit of CS formed 1 μmol citrate per min.

Histological assessment of enzyme activity

Cytochrome c oxidase staining. Cells cultured in monolayer were washed in PBS and stained for cytochrome *c* oxidase activity on a daily basis from day 3 of culture (Fig. 1). Freshly isolated cells and cells released from alginate beads were seeded onto cover slips (5×10^4 cell cm^{-2}) overnight at 37°C to attach before staining.

Attached cells were incubated in enzyme-staining mix (1 mg ml^{-1} diaminobenzidine (DAB), 1 mg ml^{-1} cytochrome *c*, 2 μg ml^{-1} catalase, 75 mg ml^{-1} sucrose in 0.05 M sodium phosphate buffer pH 7.4) for 3–3.5 h at 37°C. In the presence of cytochrome *c* oxidase, DAB was oxidised by cytochrome *c* and formed brown deposits on the mitochondrial inner membrane[36]. Cell nuclei were stained by Mayer's hematoxylin solution. Slides were air dried for 20 min before mounting in 1:1 v/v glycerol/phosphate buffer.

Gene expression analysis

Total RNA isolation. Harvested chondrocytes were washed in PBS and total RNA isolated according to the Qiagen RNeasy protocol (RNeasy Mini Handbook, pp. 25–30, http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000291). Isolated RNA was treated by DNase I (Ambion Europe Ltd, Huntington, UK). RNA concentration and purity were determined using a BioPhotometer (Eppendorf, UK Ltd). RNA integrity was evaluated by running total RNA aliquots on 1.2% denaturating agarose gels for detection of 18S and 28S ribosomal bands. Acceptance criteria of RNA samples for gene expression analysis were $>1.8 A_{260}/A_{280}$ ratio for purity and >1.5 28S/18S ratio for quality.

Collagen type I expression. Collagen type I gene expression was tested as a marker for dedifferentiation and redifferentiation of chondrocytes after monolayer culture and in 3-D alginate support[6]. Total RNA (0.5 μg) was reverse transcribed into cDNA using OneStep RT-PCR kit (cat # 210210, Qiagen, Crawly, UK). Primer sequences are given in the 5′–3′ orientation (Table I). Primers for collagen type I alpha 1 chain (*Col*I*α1*) and 18S ribosomal subunit ([18SrRNA] used as an internal control) were designed using the Primer3 software (http://frodo.wi.mit.edu/) and the Bos taurus mRNA sequences from NCBI database (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=Nucleotide&dopt=GenBank&val=BC105184) (Table I). PCR products were run on a 1% agarose tris(hydroxymethyl)aminomethane/borate/EDTA (TBE) gel and semi-quantitative analysis of DNA content was

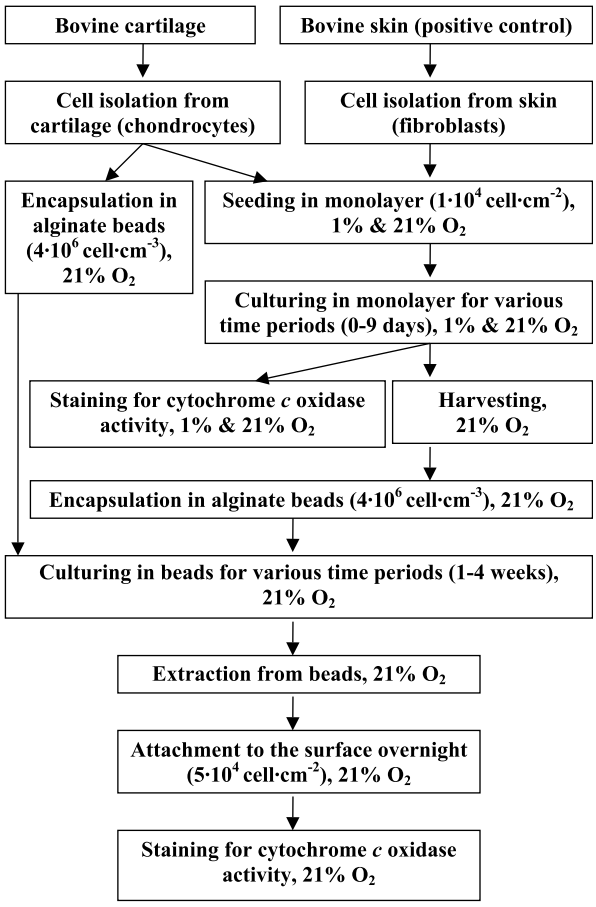

Fig. 1. Schematic showing the experimental design.

carried out using an Image Analyser. The amount of collagen type I cDNA was normalised to the amount of 18S cDNA in the same sample and then normalised to the collagen type I/18S ratio of freshly isolated chondrocytes.

COXI and COXIV expression. *COXI* and *COXIV* subunit expression levels (target genes) were evaluated by quantitative RT-PCR (qRT-PCR). Total RNA (0.1 μg) was reverse transcribed into first strand cDNA using Applied Biosystems' Reverse Transcription Reagents (cat No N8080234 Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. TaqMan gene expression was used *via*: Assays-by-Design for cytochrome *c* oxidase subunits *COXI* and *COXIV* (designed by Applied Biosystems) and assays-on-demand for 18SrRNA (Applied Biosystems cat # Hs99999901_s1). All assays used FAM™ (a trademark of Applera Corporation) as a fluorescent reporter dye and a non-fluorescent quencher dye. Sequences of primers and probes for each assay of *COXI* and *COXIV* are given in Table 1. The quantitative PCR (qPCR) reaction mix contained 10 μl 2 × Taqman universal PCR master mix (cat No. 4304437, Applied Biosystems, Warrington, UK), 1 μl 20 × concentration Taqman Assays-by-Design, 1 μl nuclease free water and 8 μl experimental cDNA sample. An ABI 7000 Prism sequence detection system (Applied Biosystems, USA) was used to perform qPCR in real time. The *COXI* and *COXIV* relative copy number was quantified by measuring the cycle threshold (Ct) and relating Ct to a standard curve. For the standard curve, a mixture of total RNA aliquots of the chondrocytes from all experimental conditions was used. The relative expression ratio (R) was calculated according to Pfaffl[37].

Statistics

R-squared (R^2) was calculated as a statistical measure of fit of a regression line to data points. The increase in overall value of cytochrome *c* oxidase activity during culture was evaluated by One-Way Analysis of Variance (ANOVA) and was considered significant when the probability value, P, was <0.05. Differences between all other variables were evaluated using two-sided unpaired and paired *t* tests and were considered significant when the probability value, P, was $^*<0.05$ and $^{**}<0.01$. All results are shown as a mean ± standard error.

Results

CELL CULTURE

Articular chondrocytes seeded in monolayer began to grow exponentially after 2–3 days of attachment. The exponential phase lasted 4 days (from day 3 to day 7) and the cells reached confluence on days 8–9 (Fig. 2a). Cell densities at the end of exponential growth phase were 0.078 ± 0.01 and 0.062 ± 0.02 million cells cm^{-2} for cells cultured under 21% and 1% oxygen, respectively. The PDL of chondrocytes under atmospheric conditions was 0.69 ± 0.07 doublings within 24 h ($n = 26$).

MITOCHONDRIAL PROTEIN

Mitochondrial protein content increased after attachment in monolayer by 4.4-fold after 3 days and by 8.0-fold at confluence relative to freshly isolated cells (Fig. 2b). The integrity of isolated mitochondria was evaluated as $85.3 \pm 2.1\%$ ($n = 30$).

CYTOCHROME *c* OXIDASE AND CS SPECIFIC ACTIVITIES

CS specific activity was proportional to mitochondrial protein (Fig. 2c) and increased with time in culture in relation to cell number (although not at level of significance (Fig. 2d) but not

Table I

Nucleotide sequences of primers and probes used for quantitative TaqMan qRT-PCR and primers used for OneStep RT-PCR. Primer sequences are given in the 5′–3′ orientation. Primers for Collα1 and 18S ribosomal subunit were designed using Primer3 programme (http://frodo.wi.mit.edu/) and Bos taurus mRNA sequence from NCBI database

Gene	Primers	Strand	Product size (bp)	TaqMan probe	Method	Reference
Collα1	aggaaactttgctccccagt accaggttcaccgctgttac	Forw Rev	841	–	OneStep RT-PCR	Personal design
18S	cattcgaacgtctgccctat agacaaatcgctccaccaac	Forw Rev	1032	–	OneStep RT-PCR	Personal design
COXI	cccgcccqtactactactactct ttccggtctgttaatagcattgtga	Forw Rev	–	ccggctgctaatacag	qRT-PCR	Applied Biosystems
COXIV	aaagttgaactgtacccctttaagt cactcatttgtgctcctgttcatc	Forw Rev	–	tcggcgaagctctcct	qRT-PCR	Applied Biosystems
18S	Not given by company Not given by company	Forw Rev	–	Not given by company	qRT-PCR	Applied Biosystems

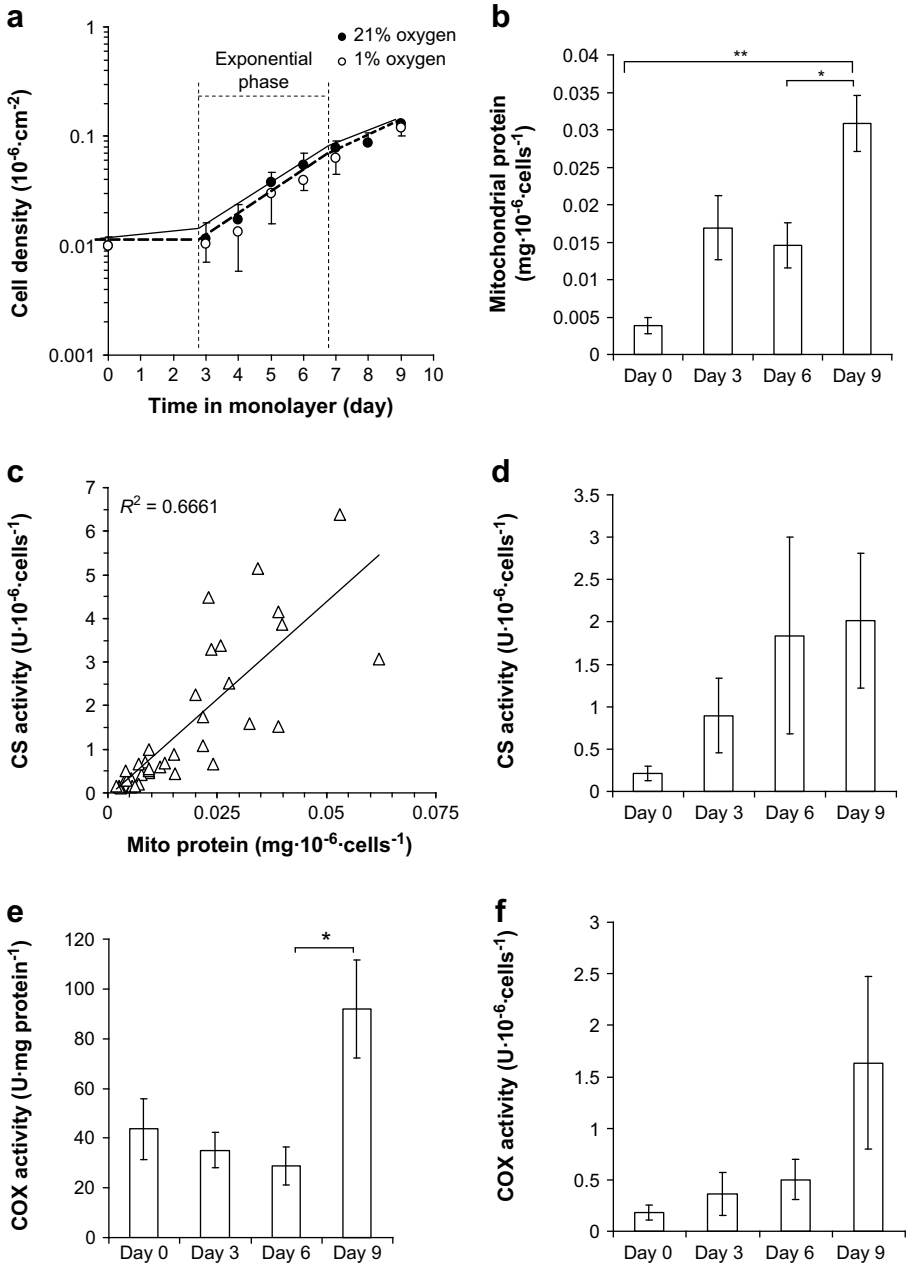

Fig. 2. Changes in chondrocyte cell number, mitochondrial protein, and CS and COX specific activities with time in monolayer culture. (a) *Growth curve*: chondrocytes were cultured in monolayer under 1% and 21% oxygen ($n = 4–6$ and $n = 8–12$, respectively, for each single point). Chondrocytes were harvested at different times during monolayer culture by incubation with 0.25% trypsin–EDTA solution, stained using trypan blue and counted under a microscope. The average number of harvested chondrocytes cultured under 21% oxygen within the exponential phase was significantly higher than for those cultured under 1% oxygen (two-sided paired *t* test, $P < 0.05$). (b) Changes in chondrocyte mitochondrial protein content with time in monolayer culture. Mitochondrial protein content increased significantly after 3 days of monolayer culture and remained high over all remaining days of culture. The number of the independent observations for single point was $n = 4–7$ ($n_{total} = 17$). (c) Dependence of the CS specific activity on mitochondrial protein content. CS activity increased with increase in mitochondrial protein content. Data were fitted to a linear regression line $y = 89.462x - 0.1023$, $R^2 = 0.67$. (d) Changes of CS specific activity of primary chondrocytes with time in monolayer culture relative to cell number. CS activity based on cell number increased markedly over the 9 days of monolayer culture. The number of the independent observations for single point was $n = 3–6$ ($n_{total} = 16$). Change in specific activity of cytochrome *c* oxidase in primary chondrocytes with time in monolayer culture relative to mitochondrial protein (e) and to cell number (f). Cytochrome *c* oxidase activity based on both mitochondrial protein and cell number increased over the 9 days of monolayer culture. The observed increase of cytochrome *c* oxidase activity on a cell basis (f) was at a statistically significant level ($P = 0.003$, One-Way ANOVA). The number of the independent observations for a single point was $n = 3–6$ ($n_{total} = 19$).

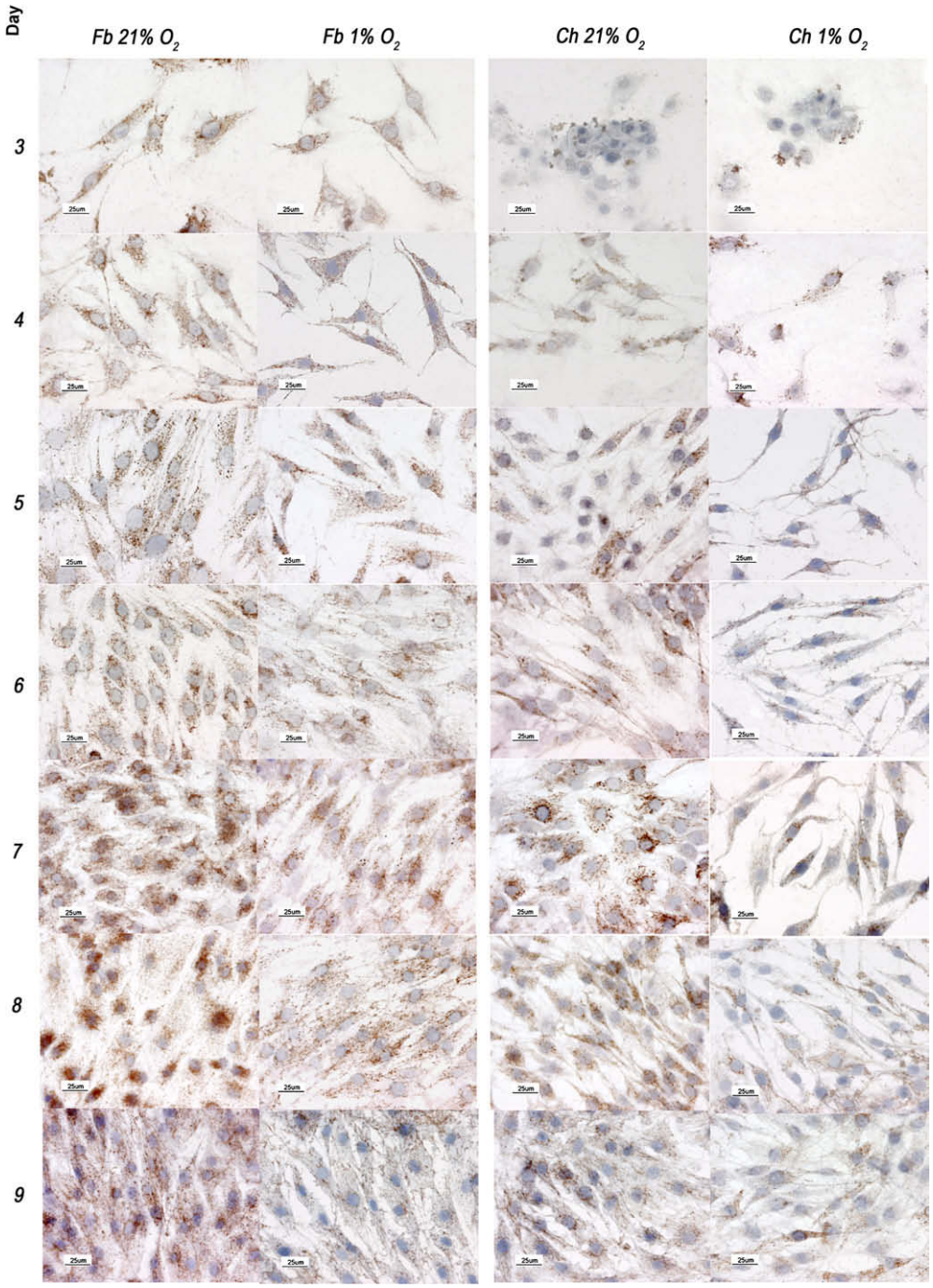

Fig. 3. Effect of oxygen tension on cytochrome *c* oxidase activity and cell shape. Fibroblasts (Fb, lanes 1, 2) and chondrocytes (Ch, lanes 3, 4) were cultured in monolayer under 21% or 1% O_2, and examined at days 3–9 for cytochrome *c* oxidase activity (brown cytoplasmic staining) and cell morphology.

in relation to mitochondrial protein (data not shown)). Cytochrome *c* oxidase-specific activity of chondrocytes in monolayer culture decreased relative to mitochondrial protein over the period of cell attachment but increased significantly at confluence (day 9) (Fig. 2e). Cytochrome *c* oxidase activity relative to cell number demonstrated an increase over the period of cell attachment, remained constant from day 3 to day 7 and then increased markedly by day 9 (Fig. 2f). Overall cytochrome *c* oxidase activity increase was found to be statistically significant (One-Way ANOVA, $P = 0.003$).

CYTOCHROME *c* OXIDASE STAINING OF CHONDROCYTES AND FIBROBLASTS

Monolayer culture under 21% and 1% oxygen

Figure 3 shows micrographs of chondrocytes expanded in monolayer under 21% and 1% oxygen for up to 9 days and stained for cytochrome *c* oxidase activity. Skin fibroblasts were used as a control. An increase in the intensity of cytochrome *c* oxidase staining with time in culture and also in the number of the chondrocytes positive for

cytochrome *c* oxidase staining was evident (Fig. 3). Freshly isolated chondrocytes and cultures within the attachment period (0–3 days) showed virtually no cytochrome *c* oxidase staining but noticeable staining was evident in chondrocytes from day 5 of culture. Fibroblasts stained markedly for cytochrome *c* oxidase from initiation of culture. Both cell types demonstrated less intense cytochrome *c* oxidase staining for cells expanded under 1% O_2 than under 21% oxygen. Chondrocytes under 1% oxygen assumed a fibroblast-like elongated shape from day 5 in monolayer; chondrocytes under 21% oxygen were still somewhat rounded until confluence (day 8/9).

Chondrocytes cultured in alginate beads for 1–4 weeks under 21% oxygen

Freshly isolated chondrocytes embedded in alginate beads and cultured under 21% oxygen for 1–4 weeks showed virtually no cytochrome *c* oxidase staining apart from a minor subset of cells demonstrating cytochrome *c* oxidase staining and/or "star-like" morphology and isolated clumps of cells demonstrating enhanced staining (Fig. 4; first column upper and lower panels, 0 days). No noticeable change in morphology and staining was observed with time in culture and by week 4, the majority of chondrocytes still exhibited a rounded morphology and the absence of cytochrome *c* oxidase staining.

Chondrocytes cultured in monolayer for 0–9 days and then returned to alginate culture for up to 4 weeks

Figure 4 shows cell morphology and cytochrome *c* oxidase staining of chondrocytes cultured in monolayer for 0–9 days (upper panel) and then returned to 3-D culture (lower panel). Results varied with time in monolayer culture. If chondrocytes were returned to 3-D culture within 4 days of initiating monolayer culture, the loss of staining and rounded chondrocyte morphology after plating as seen in freshly isolated cells was restored within 2 weeks of culture in alginate beads. Chondrocytes cultured in monolayer for longer than 4 days did not return to the original morphology after plating even after culturing for 4 weeks in beads and were still positive for cytochrome *c* oxidase staining.

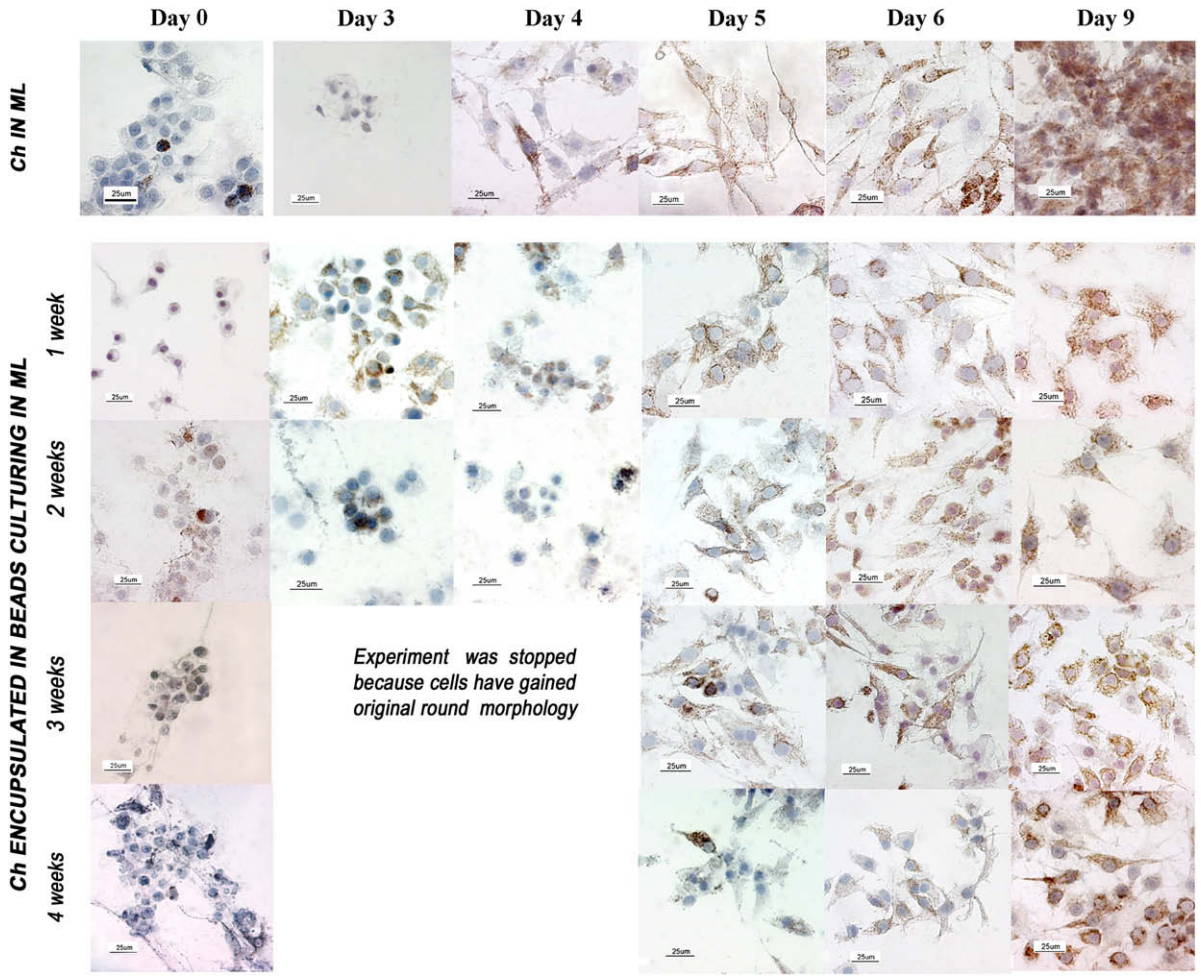

Fig. 4. Cytochrome *c* oxidase expression and morphology of chondrocytes cultured in monolayer and in 3-D alginate beads. Chondrocytes (Ch) were cultured in monolayer (ML) at 21% oxygen for up to 9 days and cytochrome *c* oxidase activity and cell morphology noted at days 0, 3, 4, 5, 6 and 9 (lanes 1–7, respectively). At each time point cells were harvested, encapsulated in alginate beads and cultured at 21% oxygen for up to 4 weeks. Cytochrome *c* oxidase activity and cell shape were measured at weeks 1–4. After week 2, no further analysis was carried out on cells harvested at days 3 and 4 as cytochrome *c* oxidase staining and cell morphology had returned to that of controls.

COLLAGEN EXPRESSION

Collagen type I gene expression increased significantly with increasing time in monolayer and than fell after cells were cultured in beads for 2 weeks, independent of time in monolayer culture (Fig. 5a,b). Collagen type I expression in cells imbedded in alginate beads remained low over the 20-day period of culture. The level of collagen II expression remained steadily high over the 9-day period of chondrocyte monolayer culture as observed by others[38,39] (data not shown).

COXI AND *COXIV* EXPRESSION

COXI expression in chondrocytes, initially low, increased markedly during the expansion period of monolayer culture (Fig. 6). After insertion into alginate beads and culturing for a further 2 weeks, *COXI* expression decreased but still remained noticeably higher than that measured initially. The level of *COXIV* expression remained more or less constant over the chondrocyte expansion period but increased noticeably at confluence (day 9), and, after restoration to a 3-D environment, fell to the level seen after isolation, independent of time in monolayer culture. The level of *COXI* expression was significantly higher than *COXIV* for all conditions tested (two-sided paired *t* test, $P < 0.05$).

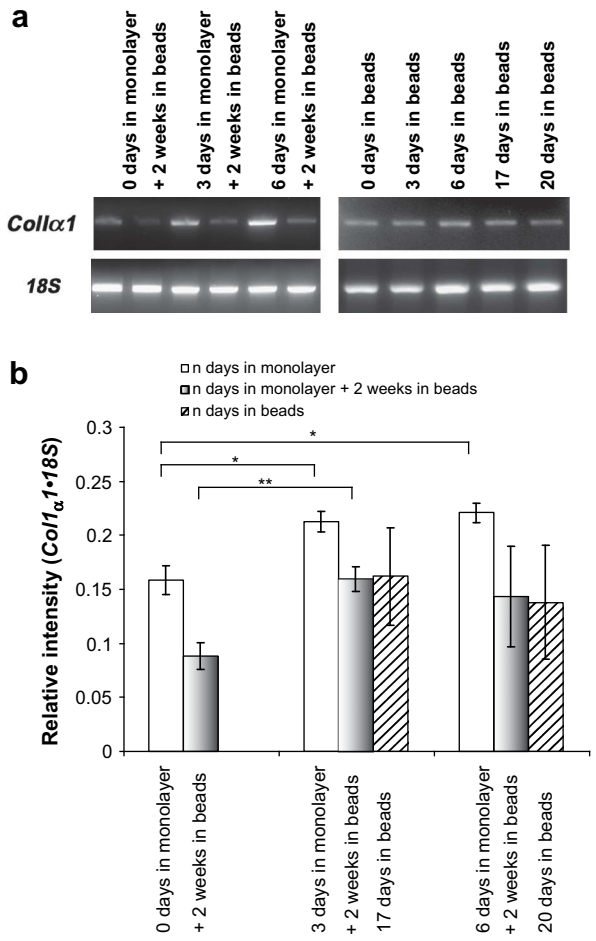

Fig. 5. Change in collagen I expression during chondrocyte monolayer culture and return to 3-D culture. Panel (a) shows RT-PCR result for *Collα1* expression in chondrocytes cultured in monolayer (first panel) and chondrocytes maintained in alginate beads (second panel). *Collα1* expression in monolayer culture increased over 6 days of culture and returned to its original level after cells in monolayer were harvested and cultured for a further 2 weeks in alginate beads. *Collα1* level in chondrocytes cultured in beads remained low over 20 days of culture: 17 days and 20 days culture corresponds, respectively, to 3 days and 6 days in monolayer followed by 2 weeks in beads. *Collα1* expression level (b) increased significantly after 3 and 6 days in monolayer culture (two-sided paired *t* test). After 2 weeks of culturing in beads, expression had returned to level observed in chondrocytes maintained in beads throughout.

Discussion

Here we found that culture in monolayer rather than purely exposure to high (21%) oxygen tension triggered mitochondrial biogenesis and cytochrome *c* oxidase expression in primary bovine chondrocytes. In freshly isolated chondrocytes, expression of cytochrome *c* oxidase, the terminal enzyme in the respiratory chain, was low (Fig. 4); we found no noticeable increase in cytochrome *c* oxidase activity when chondrocytes were cultured under 21% oxygen in 3-D culture in alginate for up to 4 weeks (Fig. 4). However culture of chondrocytes in monolayer rather than 3-D triggered synthesis and activity of cytochrome *c* oxidase within 4–5 days (Fig. 3) in agreement with other studies[22,23]. The increase in cytochrome *c* oxidase activity for cells expanded in monolayer was evident at both 21% oxygen and 1% oxygen, though the activity of cytochrome *c* oxidase was more marked at 21% oxygen (Fig. 3). Culture in monolayer also triggered an increase in mitochondrial mass per cell

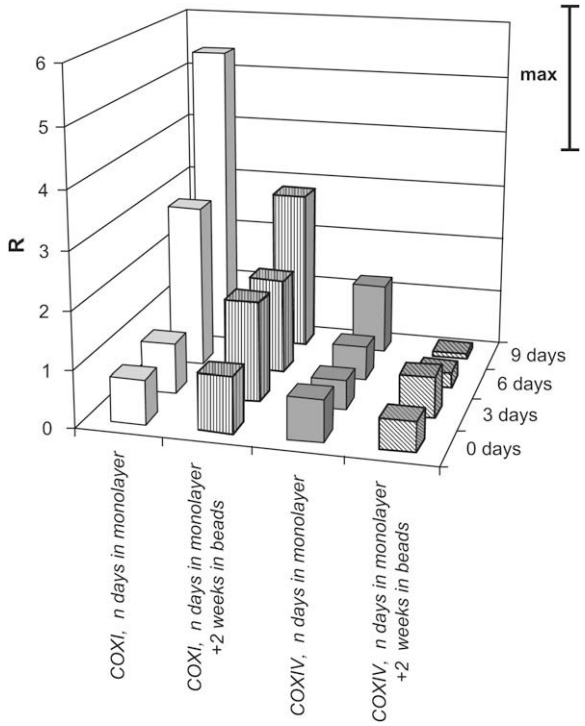

Fig. 6. Expression of the *COXI* and *COXIV* subunits of cytochrome *c* oxidase by chondrocytes in monolayer culture and after return to 3-D culture, all at 21% oxygen. Changes in expression with time of culture are more pronounced for *COXI* encoded by the mitochondrial genome than the expression of *COXIV* encoded by the nuclear genome. In addition, the time courses of change between these two subunits of cytochrome *c* oxidase are not co-ordinated.

(Fig. 2b) in agreement with earlier reports[22]. Cytochrome *c* oxidase activity/cell increased initially mainly because of mitochondrial biogenesis, but by end of the proliferation stage (>7 days in culture) cytochrome *c* oxidase activity on a mitochondrial protein basis had also increased significantly (Fig. 2e). Since most published studies on chondrocytes involving mitochondrial reactions such as free-radical signalling and apoptosis pathways are carried out in monolayer culture, such studies may significantly overestimate the role which mitochondrial-associated processes play in chondrocytes *in situ*. The physiological and pathological role of such chondrocyte mitochondrial pathways should be examined again in chondrocytes cultured in 3-D gels or *in situ* in the matrix.

The behaviour of chondrocytes when returned to 3-D culture depended on culture time in monolayer. In all cases, expression of collagen type I, which had increased in monolayer culture as expected[6] returned to levels found in freshly isolated chondrocytes within 2 weeks of return to 3-D culture (Fig. 5). However, cytochrome *c* oxidase staining only returned to levels seen in freshly isolated chondrocytes for cells cultured in monolayer for 4 days or less; for chondrocytes in monolayer for 5–9 days, staining was still evident even after return to 3-D culture for 4 weeks (Fig. 4). Chondrocyte morphology showed a similar dependence on time of culture in monolayer; after return to 3-D culture, only chondrocytes cultured in monolayer for 4 days or less assumed their rounded morphology rapidly, otherwise chondrocytes retained a fibroblastic morphology even after 4 weeks of 3-D culture (Fig. 4). Therefore it appears that some characteristics of chondrocyte dedifferentiation in monolayer such as cytochrome *c* oxidase expression and morphology occur rapidly, not after several passages, and are not reversed in 3-D culture, at least not under the culture conditions used here.

Even though cytochrome *c* oxidase activity for cells cultured at 21% oxygen was greater than for those cultured at 1% oxygen, oxygen alone was not responsible for mitochondrial biogenesis and the increase in cytochrome *c* oxidase activity (Figs. 3 and 4). The effects of oxygen may be indirect; activity of chondrocytes and of fibroblast-like cells in regard to proliferation and synthesis of collagen and other proteins varies with oxygen tension[40] and protein synthesis is markedly higher at 21% oxygen than at 1% oxygen for fibroblasts and for chondrocytes in 3-D culture and *in situ*[41–43]. Differences in rates of production of cytoskeletal proteins and of respiratory chain enzymes may thus lead to the differences between cells cultured at 21% and 1% oxygen in cytochrome *c* oxidase expression and morphology for both fibroblasts and chondrocytes (Fig. 4). The effects of oxygen are possibly modulated through its influence on energy production; chondrocytes exhibit a negative Pasteur effect and energy production rates and adenosine-5′-triphosphate (ATP) levels fall markedly as oxygen tension is reduced from 5% to 1% oxygen[11]. How glycolysis, a reaction which uses no oxygen, responds to oxygen tension is unknown, but it possibly could be adversely affected by intracellular acidification arising from a loss of oxygen free radical signalling under hypoxia[44].

While mitochondrial mass and CS activity as marker of mitochondrial mass both increased markedly during culture (Fig. 2), an actual increase in cytochrome *c* oxidase activity/mitochondrial protein was observed at the end of exponential growth phase only (Fig. 2). Cytochrome *c* oxidase gene regulation and coordination during mitochondrial biogenesis, though actively studied over the last 10–15 years, are not yet fully understood. Increase of cytochrome *c* oxidase activity could result from increase in the expression of the genes encoding cytochrome *c* oxidase (Fig. 6). It could also result from initiation of assembly of this enzyme, triggered by change of environmental parameters (such as oxygen) or resulting from the dedifferentiation process (shown here by increase in collagen type I expression (Fig. 5)). Cytochrome *c* oxidase consists of 13 subunits, three of which (*COXI*, *COXII* and *COXIII*) are encoded by mitochondrial DNA[45–49]. Assembly of this enzyme begins with a formation of a subcomplex S_2 consisting of *COXI* encoded by the mitochondrial genome and *COXIV* encoded by the nuclear genome[50]. We found that expression of *COXI* was greater than that of *COXIV* and increased at confluence, unlike *COXIV* (Fig. 6). The lack of coordination of gene expression between subunits of mitochondrial and nuclear origin and prevalence of mitochondrial transcripts above nuclear transcripts are in agreement with the results of others[22].

The increase in cytochrome *c* oxidase activity appears to coincide with a marked increase in oxygen consumption rate[25,26] possibly indicating a switch from a purely glycolytic pathway towards oxidative phosphorylation during cell expansion. Like cytochrome *c* oxidase activity, oxygen rates remain elevated in 3-D culture and could adversely affect growth of constructs of redifferentiated chondrocytes; at such high rates of oxygen consumption, oxygen would be rapidly depleted from the centre of larger constructs[25]. Understanding the link between oxygen consumption and cytochrome *c* oxidase activity and conditions which can reverse high cytochrome *c* oxidase activity in redifferentiated chondrocytes is thus of interest in regard to further development of chondrocytes cell therapies.

Conflict of interest

None of the authors have anything to disclose.

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References

1. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–95.
2. Benya PD, Padilla SR, Nimni ME. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. Cell 1978;15:1313–21.
3. Mayne R, Vail MS, Mayne PM, Miller EJ. Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity. Proc Natl Acad Sci U S A 1976;73:1674–8.
4. Diaz–Romero J, Nesic D, Grogan SP, Heini P, Mainil–Varlet P. Immunophenotypic changes of human articular chondrocytes during monolayer culture reflect bona fide dedifferentiation rather than amplification of progenitor cells. J Cell Physiol 2008;214:75–83.
5. Barlic A, Drobnic M, Malicev E, Kregar–Velikonja N. Quantitative analysis of gene expression in human articular chondrocytes assigned for autologous implantation. J Orthop Res 2008;26:847–53.
6. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
7. Bonaventure J, Kadhom N, Cohensolal L, Ng KH, Bourguignon J, Lasselin C, *et al*. Reexpression of cartilage–specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 1994;212:97–104.
8. Domm C, Schunke M, Steinhagen J, Freitag S, Kurz B. Influence of various alginate brands on the redifferentiation of dedifferentiated bovine

articular chondrocytes in alginate bead culture under high and low oxygen tension. Tissue Eng 2004;10:1796–805.
9. Zaucke F, Dinser R, Maurer P, Paulsson M. Cartilage oligomeric matrix protein (COMP) and collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes. Biochem J 2001;358: 17–24.
10. Zhou S, Cui Z, Urban JP. Factors influencing the oxygen concentration gradient from the synovial surface of articular cartilage to the cartilage–bone interface: a modeling study. Arthritis Rheum 2004;50: 3915–24.
11. Lee RB, Urban JP. Evidence for a negative Pasteur effect in articular cartilage. Biochem J 1997;321(Pt 1):95–102.
12. Otte P. Basic cell metabolism of articular cartilage. Manometric studies. Z Rheumatol 1991;50:304–12.
13. Spencer CA, Palmer TN, Mason RM. Intermediary metabolism in the swarm rat chondrosarcoma chondrocyte. Biochem J 1990;265:911–4.
14. Grimshaw MJ, Mason RM. Bovine articular chondrocyte function *in vitro* depends upon oxygen tension. Osteoarthritis Cartilage 2000;8: 386–92.
15. Guaccio A, Borselli C, Oliviero O, Netti PA. Oxygen consumption of chondrocytes in agarose and collagen gels: A comparative analysis. Biomaterials 2008;29:1484–93.
16. Stockwell RA. In: Hall BK, Ed. Cartilage. Molecular aspects. New York: Academic Press; 1983:253–80.
17. Ysart GE, Mason RM. Responses of articular cartilage explant cultures to different oxygen tensions. Biochim Biophys Acta 1994;1221: 15–20.
18. Henrotin Y, Kurz B, Aigner T. Oxygen and reactive oxygen species in cartilage degradation: friends or foes? Osteoarthritis Cartilage 2005; 13:643–54.
19. Brighton CT, Kitajima T, Hunt RM. Zonal analysis of cytoplasmic components of articular cartilage chondrocytes. Arthritis Rheum 1984;27: 1290–9.
20. Stockwell RA. Morphometry of cytoplasmic components of mammalian articular chondrocytes and corneal keratocytes: species and zonal variations of mitochondria in relation to nutrition. J Anat 1991;175: 251–61.
21. Sampson HW, Cannon M. Zonal analysis of metabolic profiles of articular–epiphyseal cartilage chondrocytes: a histochemical study. Histochem J 1986;18:233–8.
22. Mignotte F, Champagne AM, Froger–Gaillard B, Benel L, Gueride M, Adolphe M, *et al*. Mitochondrial biogenesis in rabbit articular chondrocytes transferred to culture. Biol Cell 1991;71:67–72.
23. Champagne AM, Benel L, Ronot X, Mignotte F, Adolphe M, Mounolou JC. Rhodamine 123 uptake and mitochondrial DNA content in rabbit articular chondrocytes evolve differently upon transfer from cartilage to culture conditions. Exp Cell Res 1987;171:404–10.
24. Maneiro E, Martin MA, de Andres MC, Lopez–Armada MJ, Fernandez–Sueiro JL, del Hoyo P, *et al*. Mitochondrial respiratory activity is altered in osteoarthritic human articular chondrocytes. Arthritis Rheum 2003;48:700–8.
25. Zhou S, Cui Z, Urban JPG. Change of energy metabolism after monolayer culture of chondrocytes. In: Int Cart Repair Soc, 7th World Congress, paper 4.3, 2007.
26. Heywood HK, Lee DA. Monolayer expansion induces an oxidative metabolism and ROS in chondrocytes. Biochem Biophys Res Commun 2008;373:224–9.
27. Egli RJ, Bastian JD, Ganz R, Hofstetter W, Leunig M. Hypoxic expansion promotes the chondrogenic potential of articular chondrocytes. J Orthop Res 2008;26:977–85.
28. Hopewell B, Urban JP. Adaptation of articular chondrocytes to changes in osmolality. Biorheology 2003;40:73–7.
29. Guo J, Jourdian GW, MacCullum DK. Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect Tiss Res 1989;19:277–97.
30. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, *et al*. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997;91:25–34.
31. Pon LA, Schon EA. Mitochondria. San Diego: Academic Press; 2008. p. 100.
32. Wharton DJ, Tzagoloff A. Cytochrome oxidase from beef heart mitochondria. Methods Enzymol 1967;10:245–50.
33. Milatovic D, Zivin M, Gupta RC, Dettbarn WD. Alterations in cytochrome c oxidase activity and energy metabolites in response to kainic acid–induced status epilepticus. Brain Res 2001;912:67–78.
34. Berry EA, Trumpower BL. Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. Anal Biochem 1987;161:1–15.
35. Srere PA. Citrate synthase. Methods Enzymol 1969;13:3–17.
36. Seligman AM, Karnovsky MJ, Wasserkrug HL, Hanker JS. Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB). J Cell Biol 1968;38:1–14.
37. Pfaffl MW. A new mathematical model for relative quantification in real–time RT–PCR. Nucleic Acids Res 2001;29:e45.
38. Marlovits S, Hombauer M, Truppe M, Vècsei V, Schlegel W. Changes in the ratio of type–I and type–II collagen expression during monolayer culture of human chondrocytes. J Bone Joint Surg Br 2004;86:286–95.
39. Brodkin KR, García AJ, Levenston ME. Chondrocyte phenotypes on different extracellular matrix monolayers. Biomaterials 2004;25: 5929–38.
40. Murphy CL, Sambanis A. Effect of oxygen tension and alginate encapsulation on restoration of the differentiated phenotype of passaged chondrocytes. Tissue Eng 2001;7:791–803.
41. Fermor B, Urban J, Murray D, Pocock A, Lim E, Francis M, *et al*. Proliferation and collagen synthesis of human anterior cruciate ligament cells *in vitro*: effects of ascorbate–2–phosphate, dexamethasone and oxygen tension. Cell Biol Int 1998;22:635–40.
42. Lee RB, Urban JP. Functional replacement of oxygen by other oxidants in articular cartilage. Arthritis Rheum 2002;46:3190–200.
43. Murphy CL, Sambanis A. Effect of oxygen tension on chondrocyte extracellular matrix accumulation. Connect Tissue Res 2001;42:87–96.
44. Milner PI, Fairfax TP, Browning JA, Wilkins RJ, Gibson JS. The effect of O2 tension on pH homeostasis in equine articular chondrocytes. Arthritis Rheum 2006;54:3523–32.
45. Kadenbach B, Jarausch J, Hartmann R, Merle P. Separation of mammalian cytochrome *c* oxidase into 13 polypeptides by a sodium dodecyl sulfate–gel electrophoretic procedure. Anal Biochem 1983;129: 517–21.
46. Kuhn-Nentwig L, Kadenbach B. Isolation and characterization of human heart cytochrome *c* oxidase. J Bioenerg Biomembr 1986;18:307–14.
47. Capaldi RA. Structure and function of cytochrome *c* oxidase. Annu Rev Biochem 1990;59:569–96.
48. Cooper CE, Nicholls P, Freedman JA. Cytochrome *c* oxidase: structure, function, and membrane topology of the polypeptide subunits. Biochem Cell Biol 1991;69:586–607.
49. Taanman JW. Human cytochrome *c* oxidase: structure, function, and deficiency. J Bioenerg Biomembr 1997;29:151–63.
50. Nijtmans LG, Taanman JW, Muijsers AO, Speijer D. Van den BC. Assembly of cytochrome-*c* oxidase in cultured human cells. Eur J Biochem 1998;254:389–94.